Modulation of tryptophan catabolism by human leukemic cells results in the conversion of $CD25^-$ into $CD25^+$ T regulatory cells

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Indoleamine 2,3-dioxygenase (IDO) is a novel immunosuppressive agent expressed in some subsets of normal and neoplastic cells, including acute myeloid leukemia (AML) cells. Here, we show that IDO expression correlates with increased circulating $CD4^+CD25^+FOXP3^+$ T cells in patients with AML at diagnosis. In vitro, IDO+ AML cells increase the number of $CD4^+CD25^+$ T cells expressing surface CTLA-4 and $FOX3$ mRNA, and this effect is completely abrogated by the IDO inhibitor, 1-methyl tryptophan (1-MT). Purified $CD4^+CD25^+$ T cells obtained from coculture with IDO+ AML cells act as T regulatory ($T_{reg}$) cells because they do not proliferate, do not produce interleukin (IL)-2, and inhibit naive T-cell proliferation. Coculture with IDO+ AML cells results in the conversion of $CD4^+CD25^+$ into $CD4^+CD25^+$ T cells, which is completely abrogated by 1-MT. Moreover, in mice, intrasplenic injection of IDO+ leukemia/lymphoma A20 cells induces the expansion of bona fide Treg cells by conversion of $CD4^+CD25^-$ T cells; this effect is counteracted by 1-MT treatment. These data indicate that AML cells induce T-cell tolerance by directly converting $CD4^+CD25^-$ into $CD4^+CD25^+$ T cells through an IDO-dependent mechanism. (Blood. 2007;109:2871-2877)

Introduction

Indoleamine 2,3-dioxygenase (IDO) is a key enzyme in the tryptophan metabolism that catalyzes the initial rate-limiting step of tryptophan degradation along the kynurenine pathway. 1 Tryptophan starvation by IDO consumption inhibits T-cell activation, 1,2 while products of tryptophan catabolism, such as kynurenine and O$_2$-free radicals, regulate T-cell proliferation and survival. 1,3 Thus, IDO has been shown to exert an immunosuppressive activity, and cell populations, including regulatory dendritic cells (DCs) and bone marrow (BM)–derived mesenchymal stem cells (MSCs), expressing IDO have the capacity to suppress T-cell responses to auto- and alloantigens. 3,5

A wide variety of human solid tumors express IDO. 6 More recently, we demonstrated that also acute myeloid leukemia (AML) cells, but not their normal counterparts (ie, $CD34^+$ hematopoietic stem/progenitor cells [HSCs]), express an active IDO protein, which converts tryptophan into kynurenine and inhibits allogeneic T-cell proliferation. 7 Naturally arising $CD4^+CD25^+F_{oxp3}^+$ T regulatory ($T_{reg}$) cells are known to suppress most types of immune response, 8,9 including antitumor immunity. 10-13 IDO is expressed and is functionally active in placenta, which, in turn, is infiltrated by $CD4^+CD25^+$ T$_{reg}$ cells. 14-16 Moreover, Candida albicans infection increases the number of $T_{reg}$ cells because of IDO induction in host antigen-presenting cells (APCs). 17 In human cancers, tumor-draining lymph nodes contain IDO-expressing DCs that enhance $T_{reg}$ cell function. 18 These data suggest the close relationship between IDO activity and the occurrence of $T_{reg}$ cells, 19 but the mechanism governing the generation of $T_{reg}$ cells by IDO-expressing tumors is presently unknown.

In the present study, we investigated whether the expression of IDO by AML cells may play a direct role in the development of $T_{reg}$ cells.

Materials and methods

Cells

All human samples were obtained after informed consent was signed, according to institutional guidelines. Approval was obtained from Bologna Hospital Ethical Committee. Buffy coats were obtained from healthy adults during the preparation of transfusion products. BM and/or peripheral blood (PB) samples including at least 70% leukemic cells were harvested from 76 patients with AML at diagnosis. $CD3^+$ and $CD4^+$ cells were purified from the mononuclear cell (MNC) fraction by MiniMacs high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions (purity of $CD3^+$ and $CD4^+$ cell populations was always greater than 95%). MSCs were generated from BM cells as previously reported. 20

Murine A20 and CT26 cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) or MEM (Whittaker Bioproducts) supplemented with 10% FCS (Sera Lab, Crawley Down, United Kingdom).


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RT-PCR

Polymerase chain reaction (PCR) was performed on cDNA as described elsewhere.21 Human IDO: forward 5'-ATGTTGAGGCCAAAGGT-CATGG-3', reverse 5'-AACGTGCTTCCTGGCATTG-3'; mouse IDO: forward 5'-GTGTCTGATTAGGAGGCT-3', reverse 5'-CATTTGGGCTTTCTGACTT-3' and human Foxp3: forward 5'-CCACCTTACAGGCACTTCTC-3', reverse 5'-CTTCTCTTTCTCCAGCACCA-3'. As internal control, human β2-microglobulin (β2M) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified. PCR products were separated and visualized on 2% agarose gel stained with ethidium bromide.

IDO expression and activity

Human primary AML cells as well as murine cell lines were tested for IDO expression both at the mRNA and protein levels. PCR analysis of human IDO was performed as described. For detection of IDO protein, mouse anti-IDO monoclonal antibody (mAb) (clone 10.1; Chemicon, Temecula, CA) and mouse IgG isotype antibody (Pharmineng, San Diego, CA) were used. Immunocytochemistry analysis was performed on cytospins, as previously reported.22 For IDO activity, the amount of L-tryptophan and L-kynurenine in culture supernatant was measured by high-performance liquid chromatography (HPLC) using a reverse-phase column as previously reported with modifications.4 Cells (10^6/well) were cultivated in complete medium, and supernatants were collected after 72 hours. After adding N-acetyl-tryptophan and 3-nitro-L-tyrosine (50 μM each) to the culture supernatants, the absorbance of the standard molecule (5-methyl-DL-tryptophan and 3-nitro-DL-tyrosine 1-MT; Sigma-Aldrich, St Louis, MO), as evaluated in prelimi- nary experiments, was compared with the absorbance of the supernatant samples. The percentage of optimal concentrations (1000 μM) of 1-MT was added to each well for stimulation of murine CD3+ T cells (5 × 10^5/well) cultured with 10^5/mL allogeneic CD3+ T cells (105/well) were incubated with different numbers of irradiated (3000 Gy) stimulators for 5 days. Then, cells were pulsed with 1 μCi (0.037 MBq) per well of [3H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) and assessed as previously described.23

Experimental T-cell proliferation was compared with that observed in the presence of control T cells and expressed as a percentage of inhibition.

In vivo experiments

BALB/c mice (8-10 weeks old) were obtained from Charles River (Calco, Italy) and maintained at the Istituto Nazionale Tumori under standard conditions according to institutional guidelines. Female mice were injected intraperitoneally with 10^5 A20 cells or with PBS. Mice received 1-D and L-MT (Sigma-Aldrich) in the drinking water (3.5 mL/day) at the final concentration of 5 mg/mL. After the injection (25 days), mice were killed, and the percentage of CD4+CD25+ T cells was measured in the spleen, pooled lymph nodes, and thymus. Intracellular staining of Foxp3 (FJK-165) was performed on purified CD4+CD25+ and CD4+CD25− cells according to the manufacturer’s instructions (e-Bioscience, San Diego, CA).

To test Treg cell suppressive activity, 5 × 10^4 CD4+CD25− T cells were cultured with 5 × 10^4 accessory cells (ACs) consisting in the whole 3Gy-irradiated spleen, with or without Treg cells at the indicated ratio, for 72 hours in complete medium. Anti-CD3 (1 μg/mL; ebioscience) was added to each well for stimulation. [3H] thymidine (1 μCi [0.037 MBq/well]; Amersham Pharmacia Biotech) was added for the last 10 hours of culture and measured in a microplate scintillator counter (Tomen; Wallac, Turku, Finland). To study in vivo conversion, 10 × 10^4 Thy1.1-derived CD4+CD25− cells, previously labeled with 5 μM CFSE for 15 minutes at 37°C, were transferred by tail-vein injection into recipient mice that had been inoculated intraperitonially 10 days before with 10^5 A20 cells or subcutaneously with 5 × 10^4 CT26 cells. The percentage of converted CD4+CD25+ cells in the spleen was assessed after 10 days. Cells were stained with phycoerythrin (PE)-conjugated anti-CD25, PE-Cy5, anti-CD4, and APC-Thy1.1 antibodies. The percentage of CD25+ cells over CFSE+ cells was calculated on gated Thy1.1+CD4+ cells.

Immunophenotype studies

Dual-color immunofluorescence was performed using the following panel of mAbs: PE- or fluorescein isothiocyanate (FITC)–conjugated human anti-CD3 (clone UCHT1; Pharmingen); PE- or FITC–conjugated human anti-CD4 (clone RPA-T4; Pharmingen); PE- or FITC–conjugated human anti-CD8 (clone HIT8a; Pharmingen); PE–conjugated human anti-CD25 (clone M-A251; Pharmingen); FITC–conjugated human anti–cytotoxic T lymphocyte–associated antigen (anti–CTLA-4, clone CBL 591F; Cymbus Biotechnology, Hampshire, United Kingdom); FITC–conjugated human anti–HLA-DR (clone L242; Pharmingen); PE- or FITC–conjugated human anti–HLA-DR (clone L242; Pharmingen); PE- or FITC–conjugated human anti-HLA-DR (clone L242; Pharmingen); PE- or FITC–conjugated human anti-HLA-DR (clone L242; Pharmingen).
anti–human CD45RO (clone UCHL-1; Pharmingen); and PE- or FITC-conjugated anti–human CD62L (clone MEL-14; Pharmingen). Negative controls were isotype-matched irrelevant mAbs (Pharmingen). FITC-conjugated anti–mouse CD4 (L3T4), PE-conjugated anti–mouse CD25 (PC61), and the relative isotype controls were all purchased from BD Bioscience (San Diego, CA). Cells were analyzed by using FACSscan equipment (Becton Dickinson). A minimum of 10,000 events was collected in list mode on FACSscan software.

### Statistical analysis
Results are expressed as means plus or minus standard deviation (SD). Where indicated, differences were compared using the Student t test and χ-square analysis.

### Results

**In vivo correlation of IDO expression with increased CD4+CD25+Foxp3+ T cells**

Primary AML samples (n = 76) collected at diagnosis were tested for IDO expression. Of these 76 patients with AML, 40 (52%) were positive for both IDO mRNA and protein. Moreover, IDO protein was capable to convert tryptophan into kynurenine and to reduce T-cell alloreactivity (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article).

To test whether IDO expression in patients with AML correlates, in vivo, with the frequency of Treg cells, PB samples were collected at diagnosis from patients with AML whose circulating T cells were assessable (9 IDO+ and 12 IDO−) and CD25 expression on gated CD4+ cells was evaluated. As shown in Figure 1A, the percentage of CD4+CD25+ cells was significantly increased in IDO+ AML samples compared with IDO− patients or healthy donors (P = .03). The Treg nature of CD4+CD25+ cells was evaluated by testing the expression of the winged-helix/forkhead transcription factor FOXP3, which is a widely accepted marker for naturally occurring Treg cells. In particular, highly purified CD4+ T cells were obtained from the PB of patients with AML and then evaluated for FOXP3 mRNA expression. Figure 1B shows that IDO expression correlates with FOXP3 reverse transcription (RT)–PCR positivity (P = .002). These data reveal a correlation between IDO expression and increased CD4+CD25+FOXP3+ T-cell frequency in patients with AML at diagnosis.

**IDO-expressing AML cells increase, in vitro, CD4+CD25+Foxp3+ T cells**

To investigate the role of IDO expression by AML cells on Treg cell development, we cocultured IDO+ and IDO− AML cells with highly purified allogeneic CD3+ T cells, obtained from healthy donors, in the presence or absence of the IDO inhibitor 1-MT. The viability of cells cultured in presence of 1-MT was not different from that cultured in medium alone (79% ± 12% and 82% ± 16%, respectively), as well as CD4+ and CD8+ T-cell frequencies were not modified by the addition of 1-MT (data not shown). Coculture of T cells with IDO-expressing AML cells increased both the percentage of CD4+CD25+ T cells and the surface expression of CD25 (mean fluorescence intensity [MFI] 150 ± 45 and 980 ± 30 before and after coculture, respectively; Figure 2A-B; P = .001 and P < .001, respectively), whereas coinoculation of T cells with IDO− AML cells had no effect on CD25 expression (Figure 2A-B). The addition of 1-MT to cocultures of T cells with IDO+ AML cells restored the expression of CD25 to that observed before culture, whereas 1-MT had no effect on T cells cultured with IDO− AML cells (Figure 2A-B). CD4+CD25+ T cells cultured with IDO-expressing AML cells expressed CTLA-4, which was down-regulated in presence of 1-MT (Figure 2C) as well as HLA-DR, CD62L, and CD45RO (data not shown). The described pattern of surface markers suggests that CD4+ T lymphocytes, after coculture with IDO-expressing AML cells, acquire a CD4+CD25+ Treg cell phenotype, which was markedly inhibited in the presence of 1-MT. This conclusion was corroborated by showing that CD4+CD25+ but not CD4+CD25− cells purified after coculture with IDO-expressing AML cells express FOXP3 mRNA (Figure 2D).

To investigate whether IDO activity in AML cells would result in the generation of a microenvironment that was capable per se to increase CD4+CD25+ T cells, a conditioned medium obtained from IDO+ and IDO− AML cell cultures was used to stimulate naive CD3+ T cells. Moreover, to test whether the availability of an excess of L-tryptophan within the conditioned medium could contrast IDO activity, resulting in a differential induction of CD4+CD25+ cells, the conditioned medium was generated both with standard (25 μM) and increased (150 μM) starting concentrations of L-tryptophan. According to IDO expression, only the conditioned medium collected from IDO+ AML cells had decreased tryptophan and increased kynurenine concentrations (Figure 3A,C). When we compared the contents of conditioned mediums collected from IDO+ AML cells previously cultured in 25 μM or 150 μM starting L-tryptophan, the amount of kynurenine

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**Figure 1.** IDO expression correlates with increased CD4+CD25+Foxp3+ T cells in patients with AML (A) Percentage of circulating CD4+CD25+ T cells in PB collected from healthy donors and patients with AML at diagnosis. (B) Foxp3 mRNA expression in highly purified CD4+ cells obtained from patients with AML. Data are expressed as a percentage of FOXP3+ patients. Healthy donors, n = 9; IDO+ patients, n = 9; and IDO− patients, n = 12. The data report the mean ± SD of independent experiments.

**Figure 2.** IDO expression in AML cells increased CD4+CD25+Foxp3+ T cells. (A-B) Flow cytometry analysis of CD4+CD25+ T cells before and after culture with AML cells in the presence and absence of 1-MT (1000 μM). Data are expressed as the percentage of CD4+CD25+ T cells (A) and as CD25 MFI on gated CD4+ T cells (B). Results are the mean ± SD of 10 independent experiments. (C) Surface CTLA-4 expression on purified CD4+CD25+ T cells obtained after culture with IDO-expressing AML cells in the presence and absence of 1-MT. Results are representative of 4 independent experiments. (D) Foxp3 mRNA expression by purified CD4+CD25+ and CD4+CD25− T cells obtained after culture with IDO-expressing AML cells. Total MNCs stimulated with monoclonal antibodies against CD3 and CD28 were used as positive control. Results are representative of 4 independent experiments.
AML cells. Naive CD3+ T cells were stimulated by allogeneic APCs in the presence of AML-cultured or naïve autologous T cells. As shown in Figure 4C, T-cell proliferation was markedly reduced when total T cells previously cultured in the absence but not presence of 1-MT were used (P = 0.02). This effect was markedly increased when AML-cultured CD4+CD25+ T cells were added to cell cultures (P = 0.03), whereas CD4+CD25+ gave a similar effect to that of cells cultured with 1-MT (Figure 4C). The suppressive activity of CD4+CD25+ T cells obtained after culture with IDO-expressing AML cells was dose dependent (Figure 4D).

Taken together, these data support the hypothesis that CD4+CD25+ T cells induced by IDO-expressing AML cells retain immunosuppressive activity and may be considered bona fide Treg cells.

AML cells convert CD4+CD25- into CD4+CD25+ T cells through an IDO-dependent mechanism

Coculture of IDO-expressing AML cells with CD3+ T cells resulted in the increase of CD4+CD25+ cells, which was paralleled by the decrease of CD4+CD25- cells (Figure 5A; P = 0.03). This effect could be alternatively explained considering the expansion of CD4+CD25+ cells because of active proliferation, the increased tendency to undergo apoptosis of CD4+CD25- over CD4+CD25+ T cells, or the conversion of CD4+CD25- into CD4+CD25+ T cells.

To address these points, CD4+ T cells were labeled with CFSE before the coculture with AML cells, and then monitored for the dilution of cell-associated fluorescence by flow cytometry. After coculture with AML cells, no significant proliferation was observed for CD25+ and CD25- cells, both in medium alone or supplemented with IL-2 (Figure S2). To assess the tendency of T cells to undergo apoptosis, purified CD4+CD25+ and CD4+CD25- T cells were incubated with IDO+ AML cells and stained with Annexin-V and 7-AAD (A-B). Secondary allogeneic MLRs consisting of APCs and total or purified T cells previously cultured with IDO+ AML cells convert CD4+CD25- into CD4+CD25+ T cells through an IDO-dependent mechanism.
Figure 5. AML cells convert CD4<sup>+</sup>CD25<sup>-</sup> into CD4<sup>+</sup>CD25<sup>+</sup> T cells through an IDO-dependent mechanism. (A) Flow cytometry analysis of CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells before and after culture with allogeneic IDO-expressing AML cells in the presence and absence of 1-MT (1000 μM). Results are representative of 7 independent experiments. *P < .03, experimental versus control sample. (B) Annexin-V staining and flow-cytometry analysis of CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells after culture with IDO<sup>+</sup>AML cells. Cells were analyzed at different time points. Results are representative of 3 independent experiments. (C) Purity of CD4<sup>+</sup>CD25<sup>-</sup> cells. Results are representative of 4 independent experiments. (D) Purified CD4<sup>+</sup>CD25<sup>-</sup> T cells were incubated for 7 days with normal MNCs (CTR) or AML cells in the presence and absence of 1-MT. At the end of culture, CD4<sup>+</sup> cells were gated and analyzed for the presence of converted CD4<sup>+</sup>CD25<sup>+</sup> cells. Results are the mean ± SD of 4 independent experiments. (E) Purified CD4<sup>+</sup>CD25<sup>-</sup> T cells were resuspended in the conditioned medium collected from IDO-<sup>-</sup>-AML cells and stimulated with IDO<sup>+</sup>-AML cells for 7 days. At the end of culture, CD4<sup>+</sup> cells were gated and analyzed for the presence of converted CD4<sup>+</sup>CD25<sup>+</sup> cells. Results are representative of 3 independent experiments.

Figure 6. In vivo inhibition of IDO prevents tumor-mediated expansion of CD4<sup>+</sup>CD25<sup>-</sup> T cells by blocking the conversion of CD4<sup>+</sup>CD25<sup>+</sup> cells. (A) Immunocytochemistry analysis for IDO protein in A20 cells. Image was obtained on an Olympus B×41 microscope (Olympus, Tokyo, Japan) equipped with a 40×0.75 NA objective lens and an Olympus Camedia camera. No imaging medium or solution was used. Olympus Camedia software was used for image acquisition. (B) Functional enzymatic activity. Depletion of tryptophan from the culture medium (expressed as the percentage of the starting concentration in fresh medium) by human MSCs with or without IFN-γ (positive control sample) and A20 cells. Results are representative of 3 independent experiments. (C) BALB/c mice were injected intrasplenically with 10<sup>5</sup> A20 cells (TB indicates tumor-bearing mice), and, from the day of tumor injection, they were treated or not with 1-D and L-MT (NT indicates not treated, n = 21; 1-MT, 1-MT–treated, n = 21). Two groups of mice (naive, n = 15) were not injected with the tumor but received 1-MT. Percentage of CD4<sup>+</sup>CD25<sup>-</sup> T cells among CD4<sup>+</sup> T cells in the spleen was assessed by flow cytometry analysis. Averages of data collected from experiments independently performed are reported. The data report the mean ± SD of independent experiments. (D) Intracellular Foxp3 expression in purified CD4<sup>+</sup>CD25<sup>-</sup> T cells obtained from splenocytes of tumor-bearing mice. Results are representative of 4 independent experiments. (E) Anti-CD3 mediated proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells (10<sup>6</sup>) were transferred into BALB/c mice bearing A20 or CT26 tumors. After 10 days, spleens were collected and labeled with Thy1.1, CD4, and CD25; Thy1.1<sup>+</sup> CD4<sup>+</sup> cells were gated and analyzed for CD25 and CFSE expression. Cumulative data of the CFSE<sup>+</sup>/CD25<sup>-</sup>Thy1.1<sup>+</sup> cells conversion in spleens cells are reported and represent the mean ± SD of 4 experiments.

and propidium iodide at different time points. CD4<sup>+</sup>CD25<sup>-</sup> T cells were shown to have increased apoptosis over CD25<sup>-</sup> cells, which, however, was not affected by the addition of 1-MT to cultures (Figure 5B). Taken together, these data suggest that the increase of CD4<sup>+</sup>CD25<sup>-</sup> T cells after culture with IDO<sup>+</sup>-AML cells is neither due to active proliferation nor to apoptosis.

To test the conversion hypothesis, highly purified CD4<sup>+</sup>CD25<sup>-</sup> T cells were used (Figure 5C). Figures S5 and S3 show that the percentage of CD25<sup>+</sup> cells increased over that observed after coculture with IDO<sup>+</sup>-AML cells or with control normal MNCs (P = .01). Moreover, the addition of 1-MT to cultures significantly inhibited the increase of CD25<sup>+</sup> cells induced by IDO<sup>+</sup>-AML cells (P = .03), but not by IDO<sup>-</sup>-AML cells (Figure 5D). Similar results were observed by cultivating T cells in the presence of AML-derived conditioned medium, as previously described (Figure 5E). In contrast to the results observed with total CD4<sup>+</sup> T cells (Figure S2), highly purified CD4<sup>+</sup>CD25<sup>-</sup> T cells, which had been depleted of CD4<sup>+</sup>CD25<sup>+</sup> T cells (Figure 5C), were induced to proliferate by AML cells, and converting CD4<sup>+</sup>CD25<sup>-</sup> T cells showed a significant rate of proliferation (Figure S4). Moreover, the effect on proliferation was significantly increased by the addition of IL-2 in CD4<sup>+</sup>CD25<sup>-</sup>, but not in CD4<sup>+</sup>CD25<sup>-</sup> cells. The conversion of CD4<sup>+</sup>CD25<sup>-</sup> into CD4<sup>+</sup>CD25<sup>+</sup> T cells was stable, since purified CD4<sup>+</sup>CD25<sup>-</sup> T cells obtained after culture with IDO<sup>+</sup>-AML cells did not revert to CD25<sup>-</sup> T cells once IDO<sup>+</sup> cells were removed or 1-MT was added to cultures (Figure S5). However, cultivating T cells and AML cells in a transwell resulted in a significant reduction of the conversion effect, thus suggesting that cell-to-cell contact may be required to induce this phenomenon under such experimental conditions (Figure S6). The different results obtained with AML-derived conditioned medium and those from transwell experiments may be interpreted in light of the different numbers of stimulating AML cells that were used.

These data suggest that IDO expressed by AML cells increases T<sub>reg</sub> cells by converting CD4<sup>+</sup>CD25<sup>-</sup> into CD25<sup>-</sup> T cells.

Inhibition of IDO prevents tumor-mediated expansion of CD4<sup>+</sup>CD25<sup>-</sup> regulatory cells by blocking the conversion of CD4<sup>+</sup>CD25<sup>+</sup> cells

To further explore the capacity of IDO-expressing tumors in converting CD4<sup>+</sup>CD25<sup>-</sup> into CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells, we turned to an in vivo murine system. We found that A20 lymphoma leukemia expresses IDO both at the RNA and protein levels (Figure 6A) as...
well as reduces the concentration of tryptophan from culture medium (Figure 6B). Thus, we assessed whether intrasplenic injection of A20 cells would increase the frequency of CD4+CD25+ T cells. As shown in Figure 6C, the percentage of CD4+CD25+ T cells was higher than that of non–tumor-bearing mice. Such increase was time dependent and maximal at day 25 after tumor challenge (data not shown) and, more important, was reduced by the treatment of tumor-bearing mice with 1-MT (P = .03). Purified CD4+CD25+ T cells derived from spleens of tumor-bearing mice were both phenotypically and functionally Treg cells, as shown by Foxp3 expression and the ability to suppress anti-CD3–mediated T-cell proliferation, respectively (Figure 6D-E). To investigate whether conversion may be the mechanism of Treg cell expansion in IDO+ tumor-bearing mice, CD4+CD25− T cells, purified from spleens of Thy 1.1 congenic mice, were labeled with CFSE and inoculated into Thy1.2 mice bearing IDO+ A20 tumors. For control tumor, we used the CT26 colon carcinoma, which lacks IDO expression (data not shown), but has been recently shown to expand a population of well-characterized Treg cells by conversion of CD4+CD25− into CD25+ T cells.26 In both cases a group of mice was treated with 1-D and L-MT, as previously described.6 After about 10 days from tumor challenge, spleens were collected and Thy1.1+ CD4+ donor lymphocytes were analyzed for the expression of CD25 as function of conversion and for CFSE dilution as a function of proliferation. Both A20 and CT26 tumors were capable to induce the conversion of CD4+CD25− into CD4+CD25+ T cells (Figure 6F). However, the treatment with 1-MT was effective in blocking conversion of CD4+CD25− into CD4+CD25+ T cells (P < .05) in A20-bearing mice but not in CT26-bearing mice. Moreover, no significant difference was observed as for CFSE dilution (data not shown), indicating that the expansion of Treg cells induced by IDO-expressing tumor cells was due to conversion of CD25− into CD25+ T cells in the absence of proliferation. Collectively, these data demonstrate that IDO expression by tumor is directly responsible for in vivo Treg cells expansion by conversion of CD4+CD25− into CD25+ Foxp3+ T cells.

Discussion

Since its demonstration as a potent immunosuppressive agent, IDO has been widely investigated for the induction of immunologic tolerance.27 Recently, IDO has been shown to be expressed in a wide variety of solid tumors and to prevent T-cell–mediated immunity in mouse tumor models.6 However, little is known about the mechanism(s) by which IDO-expressing tumor cells inhibit antitumor immunity. In this report, we show that IDO, which is constitutively expressed in a significant portion of patients with AML at diagnosis, directly expands CD4+CD25+ Treg cells by the conversion of CD4+CD25− T cells.

Tumor cells, including leukemia cells, are known to create an inhibitory microenvironment for the immune system,27 which could be counteracted by the optimal secretion of immunomodulatory cytokines, such as IL-12.28 Recent investigations have established the role of IDO in inducing tolerance to tumors.6,18 Published data demonstrate that the antitumor effect of IDO blockade was completely dependent on the presence of a fully competent immune system, thus suggesting that IDO acts by deregulating the host immune response. In the present study, we show that in patients with AML IDO expression is associated with an increased number of circulating CD4+CD25+Foxp3+ T cells. Such correlation is physiologic in the placenta, where decidua cells expressing IDO are fully infiltrated by CD4+CD25+ T cells,15 which have a major role in the induction of maternal tolerance against fetal alloantigen.16 Accordingly, our data demonstrate that IDO-expressing leukemia cells expand, in vitro and in vivo, a population of CD4+CD25+ Foxp3+ T cells, which functionally act as bona fide Treg cells.

Different mechanisms have been proposed for IDO-mediated immunoregulation during infection, pregnancy, autoimmunity, transplantation, and neoplasia.1 Local depletion of tryptophan and/or the production of proapoptotic kynurenines are considered responsible for the multiple effects on lymphocyte proliferation and survival after IDO induction.1,2,29 In particular, Th1 but not Th2 clones can rapidly undergo cell death in presence of low concentrations of tryptophan metabolites of the kynurenine pathway, such as 3-hydroxyanthranilic and quinolinic acids.30 In the present study, we were not able to demonstrate any role for IDO in the induction of apoptosis of T-cell subsets. Conversely, we have been able to demonstrate, in vitro, that IDO-expressing AML cells directly increase Treg cells through a mechanism of conversion from CD4+CD25− T cells. Moreover, although mice experiments cannot definitely rule out the possibility that Treg cell frequencies may be affected in vivo by different mechanisms, our results suggest that conversion may be an important pathway by which IDO-expressing tumors expand Treg cells.

Originally thought to be of restricted thymic origin, recent evidence indicates that Treg cells can also be generated in the periphery upon subimmunogenic stimuli (i.e., in the presence of suboptimal doses of antigen and APC activation),31,32 a situation resembling tumor-host interaction. Accordingly, murine tumors of different histology are capable to expand Treg cells by converting CD4+CD25− into CD4+CD25+ T cells.25 Colombo et al25 postulated that tumor-derived soluble factors, such as transforming growth factor (TGF)−β1,33,34 which are known to regulate the conversion of CD4+CD25− into CD4+CD25+ T cells,35 may play a critical role in inducing the conversion into Treg cells. Although we could not rule out that, in our system, a fraction of converting T cells may be generated in the presence of leukemia-derived production of soluble factors, such as TGF-β1, we found that T-cell conversion was completely abrogated by IDO blockade in AML cells. These data point to a direct role of IDO production in the expansion of converting Treg cells by AML cells and are in agreement with the results by Puccetti et al demonstrating that in a nontumoral mouse model, tryptophan catabolism favors the emergence of CD4+CD25−Foxp3+ Treg cells by conversion from CD4+CD25− Foxp3− cells.36 It remains to be elucidated how the modulation of tryptophan catabolism by IDO-expressing AML cells may be implicated in the conversion of CD4+CD25− cells into CD4+CD25+ cells. In particular, the accumulation of small molecules within tumor microenvironment has been recently demonstrated to affect significantly tumor-infiltrating cell populations.37 Here we show that the conditioned medium obtained from IDO+ but not IDO− AML cells was capable, per se, to induce the conversion of CD4+CD25− T cells. These data suggest that IDO+ AML cells induce a tumor microenvironment containing reduced concentrations of tryptophan and high concentrations of kynurenine, which may have a role in AML-induced expansion of Treg cells by conversion of CD4+CD25− T cells.
In conclusion, IDO production by AML cells directly increase \( T_{\text{reg}} \) cells through the conversion of CD25\(^{-} \) into CD25\(^{+} \) T cells. IDO expression can be regarded as a novel mechanism of leukemia escape from immune control and its inhibition may represent a novel antileukemia therapeutic strategy.

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References


Authorship

Contribution: A.C. was responsible for design of the research, flow cytometry, human functional tests, statistical analyses, writing of the manuscript, and review of the accuracy of the reported results; G.B., S.R., S.P., A.L., E.F., and V.S., for flow cytometry, functional tests, immunocytochemistry, and ELISAs; M.A., for molecular biology; B.V., for mouse experiments; I.D. and F.F., for HPLC analysis; M.M., A.L.H., and M.B., for critical review of the manuscript; M.P.C., for contribution to manuscript writing; and R.M.L., for contribution to the research plan and manuscript writing.

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References


Modulation of tryptophan catabolism by human leukemic cells results in the conversion of CD25− into CD25+ T regulatory cells

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